Comparative Studies on the Brown Pigments of Tobacco

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Compositional studies on tobacco have led to an examination of certain brown polymeric tobacco pigments. Initially, these pigments were shown to be combinations of chlorogenic acid and amino acids with or without rutin and iron (9, 13, 14). Subsequently, the pigments were implicated in the formation of polynuclear aromatic hydrocarbons in smoke (1). More recently, degradative studies (2) revealed the presence of a silicone, nicotine, and other bases in the leaf pigments after previous investigations (3) had shown the occurrence of these moieties in the closely related smoke pigments.

In the earlier work (13, 14), pigments of burley tobacco were the subject of examination, but later studies (1, 2) concerned the pigments of Turkish tobacco. Although pigments from both sources appeared to be somewhat similar, no direct comparisons of physical or compositional properties were made. A comparative study has now been conducted on the pigments from flue-cured, burley, Maryland, and Turkish tobacco.

The color of cured burley tobacco has been attributed to these pigments (13) and it has been postulated that they are formed during curing by a polyphenol-amino acid condensation ("Browning reaction") (5), in which the phenolic moiety is enzymatically oxidized to a quinone and the latter reacts with an amino acid. The mechanism coincides with observations made on other plant pigments and tannins. Oxidations of phenolics have been studied in tea fermentation (11), cacao curing (4), and in other vegetable tannins (7). For tobacco, the oxygen uptake in such a condensation, as catalyzed by polyphenolase, has also been studied (8). A recent review (12) lists details of other postulated couplings. In the case of tobacco, however, the recently demonstrated presence of alkaloids and a silicone in the pigment makes this postulation an oversimplification and biosynthesis of the material must involve reactions other than the classical "browning reaction."

Since the biosynthesis of these pigments is claimed to occur mainly during the curing of tobacco, different curing methods may produce pigments of different compositions and characteristics. With this additional point in mind, pigments of flue-cured, burley, Maryland, and Turkish tobacco were examined.

The pigments were obtained from blendor-ground tobacco by alkaline extraction at pH 10, as previously described (1). They contain phenolic hydroxyl and carboxylic acid groups and are precipitated from aqueous solution by acid. However, it was found more expedient to purify them in their sodium salt form and all reported analyses were performed on these salts. Purification by dialysis resulted in the loss of some low molecular weight material. The yields of pigments, on a moisture-free basis, varied from 2% to 3.4% of the leaf weight and indicate that the pigments are major constituents of tobacco. The elemental analyses were conducted on the pigment fraction with molecular weights > 3000 and are presented in **Table 1.** Iron was determined by a modification of the 1,10-phenanthrolene method (6), involving extraction of the pink complex into benzyl alcohol. Pigment material was pyrolyzed and the residue digested with fuming nitric acid. The results tabulated represent an average of six determinations, with an average deviation of about 20%. The overall results indicate a close similarity among the four types.

Other properties that were examined were infrared spectral ab-

Table 1. Analytical data on pigments

Determination %	Flue-cured	Burley	Maryland	Turkish
Yield	2.0	2.8	3.4	2.7
C	51.00	49.87	47.91	47.33
H	6.11	5.99	5.60	5.96
N	5.85	7.37	5.36	6.09
Ash	6.20	7.80	8.89	9.83
Fe	0.13	0.11	0.12	0.09
Hydrolyzable chlorogenic acid[a]	3.3	3.4	5.9	2.5

[a] *Determined as quinic acid.*

sorption, melting points, and molecular weight distributions. The infrared spectra were almost identical, showing broad hydroxyl and carboxyl absorption. Melting points were determined on both the acid and salt forms of the pigments, but were inconclusive: on heating up to 500°C, all pigments started to decompose about 300°C, leaving a final, charcoal-like residue.

Molecular weights were approximated by gel filtration on "Bio-Gel"[1] polyacrylamide, using phosphate buffer (0.002 M; pH 10) as solvent. The pattern of separation indicated that the pigments were composed of overlapping groups of molecular entities covering the whole spectrum of molecular weights from < 3000 to $\gg 100{,}000$. Consequently, a procedure was developed for obtaining a weight distribution for most of the range of molecular weights. A series of gels, having five partially-overlapping exclusion ranges, was employed. Each gel separated the pigment into one or more smaller ranges. In this manner, the pigments were fractionated into the ranges shown in **Table 2** in which the percentages of each range with respect to the total are given for a typical run. Of the fractions with molecular weights of $< 3{,}000$, the major portion of all four pigments fall in the 10,000-60,000 range. This result is in partial agreement with molecular weight determinations previously performed on a fraction of Turkish tobacco pigment (1). Material having molecular weight of $< 3{,}000$ may be due mostly to the presence of a polysaccharide, as already described (13). It is interesting to note the presence of considerable amounts of high molecular weight material, including about 5-15% in the $> 100{,}000$ range.

Table 2. Molecular weight distribution of pigments

M.W. Range	Flue-cured	Percentage distribution Burley	Maryland	Turkish
< 3000	22	15	33	20
3000-5000	11	7	4	8
5000-10,000	12	7	6	9
10,000-20,000	14	10	10	11
20,000-40,000	11	14	14	17
40,000-60,000	13	17	17	13
60,000-80,000	4	8	5	6
80,000-100,000	3	8	4	4
$> 100{,}000$	10	14	7	12

Of the several hydrolytic reactions that have been applied previously to the pigment, saponification was chosen for this comparative study. The yield of quinic acid thus obtained reflects the amount of chlorogenic acid present. If all of the quinic acid present is liberated, then this would be an indirect measure of the amount of chlorogenic acid present in the pigment structure. However, if the quinic acid moiety is bound to entities other than caffeic acid, then the amount released is still a measure of this bonding. In either case, large differences between the pigment types would be significant.

Saponification of the pigments was conducted in 10 N potassium hydroxide and the quinic acid was determined by the thiobarbituric acid method (10), with the colored complex being extracted into isoamyl alcohol. Since the results were somewhat variable, with a maximum deviation of about 30%, an average of seven determinations was used. The corresponding amounts of chlorogenic acid calculated from the data are shown in **Table 1.** On this basis, flue-cured, burley, and Turkish tobacco pigments appear to have approximately equivalent chlorogenic acid contents, while that of Maryland tobacco pigment is about twice as high.

In conclusion, this study has shown that the brown polyphenolic pigments of the four studied tobacco types are generally similar in physical properties such as color, thermal decomposition, and molecular weights. Similarity of infrared spectra and chlorogenic acid content, as inferred from the quinic acid determinations, substantiate this likeness. Expected large variations in pigment composition as a result of drastically different curing methods are not apparent. Other factors may be of importance, such as differences in chemical composition of the leaf before and during the curing process.

[1] *Mention of commercial products does not imply that they are endorsed or recommended by the Department over others of a similar nature not mentioned.*

Summary

The brown polyphenolic pigments of flue-cured, burley, Maryland, and Turkish tobacco have been examined. Comparisons of analytical, physical, and hydrolytic properties indicate that the four types of pigments are essentially similar in physical and compositional characteristics.

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